SYNOPSIS

PH.D IN FOOD SCIENCE AND NUTRITION

ASSESSMENT OF NUTRITIONAL AND FUNCTIONAL PROPERTIES OF PROBIOTIC COMPLEMENTARY FOOD MIXES FROM LOCALLY AVAILABLE CEREALS AND LEGUMES

BY

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A. Title of the Thesis

Assessment of nutritional and functional properties of probiotic complementary food mixes from locally available cereals and legumes.

B. Brief Description on the state of the art of the research topic

The process by which infant progresses from a diet composed of only breast milk to a family diet consisting of wide varieties of food which is necessary to ensure that nutrient intakes continue to be adequate for healthy growth and development throughout childhood (WHO, 2003). Fermentation is mainly by a complex microflora, which develops spontaneously with lactic acid bacteria generally overcoming the other flora as it is difficult to control, sometimes not safe since they are liable to contamination by pathogen. Therefore the need to replace the prevailing uncontrolled fermentation with a pure culture probiotic so as to obtain a consistent product quality. The probiotic complementary food mixes was designed in the present study to help restore and maintain the natural flora in the gut and support the immune system of the 6-23 months along with it reduces the diarrheal frequency as it is one of the root causes of childhood disability that contributes towards third major cause of under age five mortalities (Semwal *et al*., 2015). Since there is no such data on probiotification of complementary foods, an attempt was made to assess the acceptability of the developed probiotic complementary food mixes based on locally available cereals and legumes and to study its efficacy on the Experimental rats.

C. Definition of the problem

Diarrhoea contributes towards 3rd major cause of malnutrition, mortality and morbidity among under five children especially in the developing countries like India. According to the latest report of World Health Organisation (2020), nearly eight percentage of under age five children death in developing countries is due to diarrhoeal disease. It is, thus, clear that this disease is a major public health issue in India (Omona *et al*., 2020). United Nations International Children's Emergency Fund in 2021 revealed that deaths among under five children are mostly are caused by malnourishment (45%), Pneumonia (15%) diarrhoea (8%) and malaria (5%). Five countries namely India, Nigeria, Congo, Pakistan and China together contribute for half of the diarrhoea death among children (4.249 Million) in 2018. The National Family Health Survey (NFHS-5) shows that the prevalence of childhood diarrhoea in urban areas are 6.2% and rural areas are 7.7% in the year 2019-21.

D. Objectives

The present study entitled "**Assessment of nutritional and functional properties of probiotic complementary food mixes from locally available cereals and legumes"** has been undertaken with the following objectives:

- Development of probiotic complementary food mixes from locally available cereals and legumes
- Assessment of the physico-chemical and functional properties of the developed complementary food mixes
- *In-vivo* efficacy of developed probiotic complementary food mixes
- Study the shelf life properties of the developed complementary food mixes

E. Methodology:

Phase I:

a. Sample selection

Based on the guidelines given by Indian Council of Medical Research (ICMR) for complementary foods, different food ingredients for complementary food formulations under the present study were selected like rice (*Oryza sativa*), pearl millet (*Pennisetum glaucum*), finger millet (*Eleusine coracana*), malted wheat flour (*Triticum*), mung bean (*Vigna radiata*), soyabean (*Glycine max*), sesame seeds (*Sesamum indicum*) and pumpkin seeds (*Cucurbita sp*.).

b. Procurement of raw materials

The selected samples were procured from the local market of Coimbatore and Chennai, Tamil Nadu, India. The samples were selected for their easy accessibility, availability and high therapeutic and nutraceutical properties. The samples were stored in high density polyethylene virgin containers for future use.

c. Processing of raw materials

The raw samples (rice, pearl millet, finger millet, mung bean,pumpkin seeds, sesame seeds) were sorted and cleaned, hot air oven was used for drying the raw ingredients. The dried samples were then roasted and grinded into flour using electric grinder. The grinded flour was then sieved through different mesh size and stored in airtight containers. Soyabeans were sorted, cleaned and soaked overnight for 12 hours. Boiling of the seeds along with water was done for 30 minutes. Removal of seed coat was done manually. The seeds were then oven dried for six hours and then grinded into flour using electric grinder. The grinded flour was then sieved through different mesh size and stored in airtight containers.

d. Development of the ARF

Wheat grains were soaked in water for 12 hour and then kept in muslin cloth for germination for 72 hours. Hot air oven dryer was used for drying for 12 hours, then grinded into flour using electric grinder, sieved and stored in airtight container.

Phase II:

Determination of nutritional characteristics of raw ingredients:

The proximate composition i.e moisture, crude fat, crude protein, crude fibre and total minerals were determined by A.O.A.C methods. The available carbohydrate content was estimated by the method of Hedge and Hofreiter (1962). Mineral elements like iron(Fe) was determined according by Ranganna (1997) by using spectrophotometer, zinc (Zn) content was estimated in the sample by using Atomic Absorption Spectrophotometer, calcium (Ca) was determined by using flame photometer according to the method A.O.A.C, Phosphorus (P) was determined by Photometric method by Wheeler and Ferrel (1971) and tannins were determined by modified Vanillin-HCL in methanol method by Broadhurst and Jones(1978).

Phase III:

a. Formulation and standardization of complementary food mixes using ARF:

Based on the guidelines given by Bureau of Indian Standards (2006) for complementary foods, six different compositions (T_1 to T_6) for complementary food mixes under the present study were formulated. The formulation with the highest energy density (ED) was subjected to further Probiotification. The total energy content of the formulations were determined by adding the estimated value of carbohydrate, protein and fat of all the ingredients for each test samples and then multiplying with a constant factor of 4 for carbohydrate and protein and 9 for fat. A total of six test samples were formulated using different level of incorporation. The energy density value of the formulations should be between 300– 550 kcal per 100g of sample.

Total energy (kcal/100 g) = [(% available carbohydrates × 4) + (% protein × 4) + (% fat × 9)]. Standard principles outlined by ICMR (2010).Formulation T4 with rice and mung bean had the highest ED value 1830KJ (437.50Kcal). Formulation T6 with finger millet and mung bean had second highest ED value 1855 KJ (443.49 Kcal).

Phase IV:

a. Probiotification of selected complementary food mixes:

The probiotic cultures were obtained from National Collection of Dairy Cultures (NCDC), ICAR – National Dairy Research Institute Karnal, Haryana. The probiotics selected were *Lactobacillus fermentum* (NDRI-141), *Lactobacillus casei* (NDRI 297), *Lactobacillus rhamnosus* (NDRI-353) and *Lactobacillus plantarum* (NDRI-375). Nutrient Agar and MRS (de Man Rogosa Sharpe) broth were selected as the suitable medium for the growth and culturing of probiotic bacteria. Culture was incubated at 37^{o}C temperature for 24 hours under appropriate gaseous conditions. Four conical flasks of 50 ml were used for the preparation. Mixing of pure culture from agar plates in 50 ml conical flask with 25 ml of MRS broth and incubating in a BOD incubator for 24 hours at 37^{o} C. Centrifuging bacterial cultures grown in broth at 5000rpm for 15 minutes to obtain pure palate. The bacterial cultures grown in broth were then centrifuged once at 5000 rpm for 15 minutes to obtain pure palate. The supernatant was thrown and pure palate were taken and added with 10 ml of normal saline. The density of bacterial cell suspension/inoculum (stored pure palate) was compared to the freshly prepared 0.5 McFarland turbidity standard scale by holding them both in front of a light against a contrast background. The density of inoculum was adjusted by diluting it with more sterile normal saline if needed. Submerged fermentation (1:3) was carried out by adding 297 ml of sterile distilled water aseptically in 500 ml of conical flask and mixed thoroughly with 100 g of mixes separately (T_4 and T_6) in a homogenizer and sterilized at a temperature of 121^{o}C for 20 minutes. The fermentation jars were cooled and inoculated at 1% of 10^{8} CFU m/L, tightly covered with aluminium foil to keep it away from contamination and kept undisturbed in a BOD incubator for 24 hours at 37^{o} C and then freeze dried for 72 hours and packed in airtight containers. The mixes after Probiotification were named as CFM I (T_4) and CFM II (T_6).

b. Acceptability trials of developed probiotic complementary food mixes.

For evaluating the acceptability of the developed formulations, score card method was selected. In the present study, a score card consisting a table utilizing the Hedonic ratings of nine point scale (Peryam and Pilgrim, 1957) from like extremely to dislike extremely. The acceptability of the formulated complementary mixes was assessed by score card for sensory characteristics. Semi trained panel i.e a total of 30 staff members and students from the Department of Food Science and Nutrition, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore evaluated the prepared samples for acceptability trials. The IHEC Approval number is AUW/IHEC/FSN-21-22/XPD-33.The product has been

patented under the name "**A process of preparation of probiotic complementary food mix and product thereof**" with application number **202241007163**".

Phase V:

I. Analysis of physical and nutritional composition of complementary food mixes

a. The morphological and biochemical characterization of bacterial cultures were done by the methods described by Giuliano *et al.,* (2019).

b. Resistance to gastric acidity of food mixes at different ph values at different exposure time were done by standard protocol given by Awan and Rahman (2005).

c. Bile acid resistance was done by Awan and Rahman (2005)

d. Antimicrobial activity was carried out by disc diffusion method (Daoud *et al.,* 2015).

II. **Analysis of physical composition of complementary food mixes**

Physical characteristics of the developed complementary food mixes: Bulk density was measured by the method suggested by Lewis, (1987), Swelling capacity were also determined with the method suggested by Appiah *et al*., (2011), Viscosity was measured by the modified method of Hallic and Kelly (1959) by using Visco basic plus viscometer, Water and fat absorption capacity of the flours was determined by the method of Sosulski *et al*. (1976). The sedimentation volume was described according to AACC (2002). Dispersibility is calculated by Kulkarni *et al*., (1991). Texture was analysed by the modified method given by Mantihal *et al.,* (2019) by using a TA.XT-Plus Texture analyser.

a. Proximate composition of the probiotic complementary food mixes were done according to the methods stated in Phase II.

b. Phytochemicals like flavonoids, alkaloids, terpenoids, glycosides, tannins, phenolic, and saponins were analyzed by qualitative chemical method and quantitative method Gierschner, *et al* and Latif *et al*., (2020).

c. Mineral composition of Probiotic complementary food mixes were done by A.O.A.C methods.

d. Free radical scavenging activity was measured by using DPPH method according to Vani *et al.,* (1997).

e. *In vitro* starch digestibility was assessed with modification of the methods given by Som *et al.* (1992). *In vitro* protein digestibility was determined according to the modified method described by Walter *et al.,* (1983).

III. *In vivo* efficacy for quality of the developed complementary food mixes

The experiments were conducted in the Animal house, Department of Biochemistry, Avinashilingam Institute for Home Science and Higher Education for Women. Coimbatore. The IAEC Approval number is AIW:IAEC.2020:FSN:01.

a. The impact of feeding probioticated CFM I and CFM II on the gut microflora of rats through fecal microbial count were done through the standard protocol described by Barai *et al.*, 2018.
b. Net protein ratio (Bender and Miller, 1953) is the simplest procedure to evaluate the protein quality. It focuses on weight loss of protein free group to the weight gain of group fed with protein diet.
c. The food conversion efficiency will be calculated as per method of Shingari and Sapra(1991).
d. The blood glucose level will be measured using SIEMENS Glucose *in vitro* diagnostic kit.
e. The total blood protein levels will be measured by using SIEMENS Total Protein *in vitro* diagnostic kit.

Phase VI:

I. **Shelf life properties of the developed complementary food mixes**

a. Total bacterial count was analyzed by the Standard operating procedure for the enumeration of micro-organisms Colony Count Technique at 35°C.
b. Peroxide value of the samples was determined by following AOAC (1975) method.
c. Free fatty acid content of the samples were determined by following AOAC (1970)

II. **Data collection & Statistical analysis**

All data were analyzed using SPSS v.17.0 for Windows (SPSS Inc., Chicago, IL). Statistical significance was set at $P< 0.05$ and $P<0.01$

F. The salient findings of the study are as follows:

- The moisture content was found to be highest in malted wheat flour 4.01±0.10 g among cereals, soyabean 5.02±0.10 g among pulses and sesame seeds 3.30±0.10 g among oil seeds.
- The available carbohydrate was found to be highest in finger millet 88.50±0.015 g among cereals, mung bean 73.00±.005 g among pulses and sesame seeds 25.40±.005 g among oil seeds.

- The crude protein was found to be highest in 30±.005 g in malted wheat flour among cereals, soyabean 39.28±.005 g among pulses and pumpkin seeds 30.10±.001 g among oil seeds.
- The crude fat content was found to be highest in malted wheat flour 9.8±0.002 g among cereals, soyabean 10.48±0.13 g among pulses and pumpkin seeds 51.90±0.15 g among oil seeds.
- Finger millet had the highest calcium 330.01±0.010 mg, iron 6.71±0.010 mg and zinc 3.00±0.005 mg content among cereals, Phosphorus was found to be highest in malted wheat flour 811.00±0.005 mg content. Among pulses, soybean had the highest calcium 220.01±0.010 mg, iron 11.01±0.005 mg, zinc 3.31±0.010 mg and phosphorus 675.01±0.015 mg content. Among oil seeds, sesame seeds had the highest calcium 1330.01±0.010 mg, iron 8.91±0.010 mg, zinc 12.11±0.010 mg and phosphorus 551.69±0.010 mg content.
- The phytate content was found to be highest in finger millet 699±0.010 mg/100g, oxalates 39±0.005 mg/100g and tannins 280±0.010 mg/100 g was found to be highest in pearl millet.
- The highest energy density values i.e T4 with rice and mung bean with 1830KJ (437.50 Kcal) and T6 with finger millet and mung bean with 1855 KJ (443.49 Kcal) were selected for further analysis.
- The protein content 12.24±0.010 percentage, Tannins 10.91±0.005 percentage, water absorption capacity 0.356±0.005 ml/g and bulk density 2.58±0.005 g/ml was found to be highest in T6.
- The viable number of probiotic bacteria was found to be highest in T4 with Lactobacillus plantarum (CFM I) i.e 6×10^{8} and T6 with Lactobacillus casei (CFM II)i.e 7.2×10^{8} .
- Resistance to gastric acidity of food mixes at different ph values at different exposure time was found to be highest in CFM I at P.H 3 at 3 hours i.e 4.5×10^{8} and at 6 hours 3.9×10^{8} and CFM II at P.H 3 at 3 hours i.e 5.6×10^{8} and at and at 6 hours 5.5×10^{8} .
- Tolerance to bile salts was found to be highest in CFM I, 0.5 oxgall i.e 5.5×10^{8} percentage and CFM II, oxgall 6.9×10^{8} percentage.
- Out of the four formulations prepared, the probiotic complementary food mix developed from Finger Millet (CFM II) was best scored in colour, flavour, appearance and overall acceptability. CFM I developed from rice scored highest in consistency.

- The bulk density (g/ml) 0.54±0.005, Sedimentation volume (ml) 5.80±0.015, Water holding capacity (%) 220±1.00, Fat holding capacity (%) 237±1.00 ,Dispersibility (%) 64.66±1.15 of CFM I was found to be highest among the four formulations. The Swelling capacity (%) 19.00±0.10 of CFM II was found to be highest among the four formulations.
- The viscosity (cP) was found to be lowest in CFM II 951.00±1.00 at 12 rpm, 566.16±1.04 at 30 rpm and 401.20±1.08 at 60 rpm. A low viscosity value with high nutrient content is a desirable characteristic of complementary foods (Ariahu *et al.,* 1999).
- The Adhesiveness (J) 4.16±.03, stickiness -5.63±0.06 and Stringiness (mm) 3.30 ±0.04 was found to be highest in CFM II. Resilience 0.35±.010 was found to be highest in standard commercial sample.
- The moisture (g) 3.85±.010, available carbohydrate (g) 82.69±.0.10, crude protein (g) 19.90±.0.09, crude fat (g) 6.60±.05, crude fibre (g) 6.12±.07, total minerals (g) 1.81±..05, total energy 490 kcal of CFM II was significantly higher than CFM I.
- The nutraceuticals content of CFM II was significantly higher than CFMI. The nutraceuticals content of CFM II are given as follows, flavonoids content (mg/100g) 130±0.95, Alkaloids (mg/100g) 41.06±.51, Cyanogenic glycosides (mg/100g) 26.53±0.45, tannins(mg/100g) 6.29±.06, oxalates (mg/100g) 0.55±.01, trypsin inhibitor (mg/100g) 4.20±0.25, saponins (mg/100g) 13.41±.07, phytate (mg/100g) 1.6±.03.
- The mineral composition of CFM II was significantly higher than CFM I. The mineral content of CFM II (in PPM) values are given as sodium 5.72±0.24, potassium 432.38±0.33, calcium 259.58±0.38, magnesium 6.85±0.30, manganese 15.48±0.42, iron 13.65±0.522, zinc 4.84±0.14, copper 14.15±0.217, phosphorus 55.11±0.99.
- The free radical scavenging activity of CFM II was significantly higher than CFM I. The free radical scavenging activity of CFM II was 74.3 percentage and CFM I was 72.2 percentage.
- The protein digestibility of the formulated complementary food mixes ranged from 70.26±0.37 percentage in CFM I to 73.46±0.50 percentage in CFM II and were statistically different at ($p \leq 0.05$) from each other.
- The reduction of diarrhea was found to be highest in positive control fed with Loperamide 3mg/kg 70.68 percentage. Among the test sampes fed it was found to be significantly higher in CFM II i.e 68.88 percentage and CFM I i.e 64.84 percentage.

- Impact of feeding probioticated on the gut microflora of experimental rats was found to be increasing till the 4^{th} day in both the test samples ie CFM I 7.8 and CFM II 9.2. The probiotic count were statistically different at ($p \leq 0.05$) from each other.
- The net protein ratio was found to be highest in reference diet fed with casein diet i.e 31.37±0.28. Among the test diet the CFM I had net protein ratio of 26.11±0.67 and CFM II had net protein ration 30.17±0.48 which was significantly higher than CFM I.
- The food conversion efficiency was found to be lowest in CFM II i.e 0.18±.015, CFM I 0.22±0.20. A low FCE is an indication of a high quality feed.
- Blood glucose levels in experimental animals was found to be statistically different at ($p \leq 0.05$) from each other till 21^{st} day. The blood glucose levels was found to be 102.9433±0.91 for group I, 88.4433±1.38 for Group II and 91.1667±1.25 for Group III.
- Total blood protein levels in experimental animals was found to be statistically different at ($p \leq 0.05$) from each other till 21^{st} day. Total blood protein levels was 7.35±0.12 for Group I, 12.51±0.48 for Group II, 13.29±0.611 for Group III.
- The total bacterial count for CFM I was 5.18×10^8 and CFM II was 6.18×10^8 till 60^{th} day of storage. The total bacterial count were statistically different at ($p \leq 0.05$) from each other.
- Free fatty acid for CFM I was 4.29±0.55 percentage and CFM II was 4.05±.05 percentage for 70^{th} day and was not fit for consumption. No remarkable change was observed up to 2 months of storage which may be due to the packing of the flours in polyethylene bags, sealed and stored in cool temperature (4 degree).
- The peroxide values for 70^{th} day for CFM I was 6.95±0.05 m moles/kg fat and CFM II was 7.05±0.04 m moles/kg fat. The peroxide value of the developed products was below the standard values prescribed by Prevention of Food Adulteration Rule, 1991.

G. Conclusion

The present work focused on nutritional enhancement of complementary food mixes from locally available cereals and legumes. Developed complementary food mixes are nutritionally rich baby food developed using locally available food materials that are low cost and from all the food groups i.e. cereals, pulses, oilseeds. Six different (T_1 to T_6) compositions were formulated out of which T_4 and T_6 had the highest energy density values adding ARF. The addition of ARF to existing complementary food for malnourished and growth-faltering young children is a simple and effective means to change the consistency of

the food to facilitate its ingestion by young children and thus optimize their dietary (and energy) intake. Fermentation of both the mixes (T_4 and T_6) was carried out to enhance the bioaccessibility and bioavailability of nutrients and improves organoleptic properties as well as in extending the shelf life. The fermentation process was carried out in aseptic environment in Laminar Air flow using safety measures such as gloves. Freeze drying was done at a temperature of -53^0C was done for the samples to avoid contamination and dried samples were then stored in polyethylene bags. The mixes after Probiotification were named as CFM I (T_4) and CFM II (T_6). The mixes after probiotification were further accessed for nutritional and functional characteristics. Out of the two formulations CFM II was found to be highly acceptable and possessed high nutritional and functional contents, free radical scavenging activity and nutraceutical potentials. The *in-vivo* studies stated that both the product were effective in reducing the episodes of diarrhea along with it increasing the beneficial bacteria in the gut microflora, increasing the total protein and total weight and maintaining the blood glucose. The shelf life studies stated that both the products are good for consumption for a period of 60 days when kept under refrigerated temperature (4^OC). Dissemination of this technology in to the rural areas on large scale will be helpful to the rural farm women to feed their children a nutritionally balanced feed. The probiotic fermentation resulted in favourable changes in nutritional profile of complementary food mixes resulting in increase in nutritional quality and decrease in antinutrient contents. Hence, it can be recommended that the probiotic fermentation technology possess a good potential to develop nutritionally enriched complementary mixes.

H. **Paper Published:**

1. Manisha Sharma and Kowsalya, S., Comparative Study on Different Drying Techniques for determining the Moisture Content of Cereals /Millets and Pulses, *The Indian Journal of Nutrition and Dietetics,* Supplement - 2, January - March 2021. UGC CARE list-Sciences S. No. 392.

2. Manisha Sharma and Kowsalya, S. A review on: functional foods and its growing trend in India, Journal of *Xi'an University of Architecture & Technology,* Volume XIII, Issue 8, 574-584. Scopus Indexed. Issn No. : 1006-7930

I. Paper Presented

1. Presented in Home science association meet on "Functional Foods: The Need Of The Hour" on 22nd - 23rd January 2020
2. Presented in International web conference on food technology and nutrition-prospects for health on "A comparative study on different drying techniques for determining the moisture content of cereals/millets and pulses" on 28th – 29th January 2021

J. Recommendations

- The product as a published patent technology of product development should be transferred to industries so that this will reach the community.
- To create awareness on homemade complementary food mixes prepared using locally available cereals and legumes, popularization can be done through social media aids.
- The research work has used only *in vitro* models to assess the protein digestibility of the developed complementary food mixes. In future work, new studies can be done at molecular and cellular level to understand the protein digestibility of weaning foods which helps to improve infant health**.**

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